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# Epidemiologic Study of Influenza Infection in Okinawa, Japan, from 2001 to 2007: Changing Patterns of Seasonality and Prevalence of Amantadine-Resistant Influenza A Virus▽

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**To clarify seasonal influenza patterns and the prevalence of amantadine-resistant influenza A viruses in Okinawa, located at the southern extremity of Japan in a subtropical climate, we conducted a laboratory-based study of influenza virus infections from 2001 to 2007. The annual outbreaks tended to show two peaks in Okinawa, in summer and winter, although the main islands of Japan, located in a temperate climate area, showed only winter influenza activity. Epidemic types and subtypes in Okinawa mostly matched those on the main islands of Japan in winter and those in Taiwan in summer. Rates of amantadine resistance dramatically increased, from 7.3% in the November 2002-to-March 2003 season to 90.0% in summer 2005, and a similarly high rate of resistance continued for the rest of the study period. Phylogenetic analysis of the hemagglutinin gene of A/H3N2 isolates collected from 2002 to 2007 revealed a monophyletic lineage that was divided into four period groups. Each group included amantadine-sensitive and -resistant viruses within independent clusters. In the November 2005-to-March 2006 season, all of the amantadine-resistant viruses were clustered in clade N, with dual (position 193 and 225) amino acid mutations in their HA1 subunits. In 2005, clade N amantadine-resistant viruses existed in Okinawa several months before the circulation of this clade on the main islands of Japan. In conclusion, surveillance in Okinawa to monitor influenza virus circulation is important for elucidating the dynamics of virus transmission in a border area between temperate and subtropical areas, as Okinawa is one of the best sentinel points in Japan.**

Influenza is generally associated with seasonal (winter) epidemics in temperate regions, but a year-round pattern with a rainy season peak is observed in the tropical regions (7, 36). A recent report on antigenic and genetic analyses of the hemagglutinin (HA) of influenza A/H3N2 viruses revealed that these viruses have continuous circulation in East and Southeast Asia via a region-wide network of temporally overlapping epidemics and that epidemics in the temperate regions are seeded from this network each year (24). Our group has demonstrated previously by a spatial analysis of epidemiological data from the National Influenza Surveillance in Japan that influenza epidemics spread in concentric circles from west-central Japan to northeastern Japan (30) We also reported that the emergence of new strains in Vietnam was detected earlier than that in Japan (16). Therefore, we assume that influenza spread from neighboring countries in East and Southeast Asia to Japan, as was suggested by a previous report (24).

Recent studies highlighted an extreme increase in the prevalence of amantadine-resistant influenza A/H3N2 viruses in Asian countries including Japan and in Oceania and North America after 2005 (2, 4–6, 16, 26–28, 39). Amantadine has been used to control influenza infections (21), and its use is associated with the rapid emergence of drug resistance mutations at codons 26, 27, 30, and 31 of the M2 protein gene (10, 22, 33). However, the recent amantadine-resistant strains apparently spread regardless of the drug pressure, are characterized by signature amino acid substitutions, Ser193Phe and Asp225Asn, in HA, and belong to a phylogenetic group named clade N (2, 6, 16, 26–28). These clade N viruses were first detected in Southeast Asia and Oceania in May 2005 (2, 16) and spread to the main islands of Japan several months later (25–27).

Okinawa has unique characteristics in terms of geographical location and climate in Japan. It is located at the southern extremity of Japan, close to Taiwan, and is the only prefecture in Japan situated in a subtropical climate zone. We took notice of these peculiarities and conducted a laboratory-based influenza study to clarify the seasonal influenza patterns and the prevalence of amantadine-resistant influenza A viruses from January 2001 to March 2007 in Okinawa. We also carried out a longitudinal study of genetic changes in the HA molecule of the amantadine-sensitive and -resistant A/H3N2 strains.

## MATERIALS AND METHODS

Okinawa Prefecture is located at the southern extremity of Japan and consists of 160 islands, including 49 inhabited islands. It has more than 2,000 mm of annual rainfall and a yearly average temperature of approximately 22°C. Okinawa belongs to the subtropical climate zone and is close to Taiwan, which has the same climate as Okinawa. The prefecture is one of the most famous tourist spots in Japan, and several million tourists from the main islands of Japan and foreign countries visit Okinawa each year. Among the foreigners, travelers from

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Taiwan were the largest group and accounted for 50 to 70% from 2001 to 2006 (2006 Okinawa Prefecture tourism report [in Japanese; http://www3.pref.okinawa.jp/site/view/contview.jsp?cateid=233&id=14738&page=1]).

**Epidemiological information.** In Japan, a new infectious disease control law was enacted in April 1999, and since then, influenza and influenza-like illness (ILI) have been under systematic surveillance (http://www.nih.go.jp/niid/index.html). An ILI case is defined on the basis of the sudden onset of fever over 38°C, respiratory symptoms, and other systemic symptoms (fatigue, headache, or myalgia). Positive results from influenza rapid test kits are used to diagnose influenza. Both influenza and ILI cases are eventually filed under the classification "influenza" in the scheme. Okinawa Prefecture assigns 58 pediatric and internal medicine clinics and hospitals to collect epidemiological information under the National Influenza Surveillance program. In this study, the monthly number of influenza cases in Okinawa was obtained from the National Influenza Surveillance data published in the Infectious Disease Surveillance Center's electronic archives and on the center's web site during the period from January 2001 to March 2007 (http://idsc.nih.go.jp/idwr/index.html). The local circulation of influenza was regarded as "active" if more than two influenza patients per sentinel site in the prefecture per week were reported or more than two influenza virus isolates per week were recovered at the microbiology division of the Okinawa Prefectural Institute of Health and Environment.

**Virus isolation.** Under the National Influenza Surveillance, nasopharyngeal specimens from influenza patients were collected at 5 of the 58 sentinel medical facilities before the initiation of any influenza therapy. The samples were kept at 4°C at the medical facilities and transferred to the microbiology division of the Okinawa Prefectural Institute of Health and Environment for influenza virus isolation. Samples (200 μl) of supernatants from cultures of nasopharyngeal swabs were inoculated into Madin-Darby canine kidney (MDCK) cells, with 1 ml of maintenance medium, containing trypsin at a concentration of 5.0 μg/ml, per well in 24-well plates. The plates were incubated at 35°C in a 5% $CO_2$ atmosphere for 1 week to assess cytopathic effects. All of the isolates were typed and subtyped by the hemagglutination inhibition assay. Ferret panel sera, which were provided by the National Institute of Infectious Disease, Tokyo, Japan, was used in the hemagglutination inhibition assay. Goose red blood cells were used until 2002, but guinea pig red blood cells were used beginning in December 2003. Influenza virus isolates were sent from the Okinawa Prefectural Institute of Health and Environment to the Department of Public Health, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan, for further virological investigations.

**Amantadine susceptibility testing.** Amantadine susceptibility testing using the 50% tissue culture infective dose ($TCID_{50}$) per 0.2 ml, a previously described phenotypic assay for amantadine susceptibility (18), was employed for all influenza A/H1N1 and A/H3N2 virus isolates. Two series of 10-fold dilutions of viruses from cytopathic effect-positive cultures were plated in triplicate into 96-well microplates with confluent MDCK cells; one dilution series contained a final amantadine concentration of 1.0 μg/ml, and the other dilution series did not contain the drug. After incubation for 48 h at 37°C, virus titers for amantadine-containing and -free rows in triplicate were calculated by the Reed-Muench format from the last dilutions at which all cells were infected (23). The susceptibility test results were assessed as interpretable if the virus titers in amantadine-free rows exceeded 2.5 $\log_{10}$ $TCID_{50}$/0.2 ml. Amantadine-resistant strains were identified when a difference of less than twofold in the log $TCID_{50}$/0.2 ml titer between series of rows with and without the drug was observed.

**RNA extraction and PCR.** Viral RNA was extracted from 100 μl of culture supernatant by using an Extragen II kit according to the instructions of the manufacturer (Kainos, Tokyo, Japan). Reverse transcription to create cDNA was performed using the influenza A virus generic primer Uni12 as reported elsewhere (9). PCR was performed using M2 gene-specific primers to amplify a 231-bp product covering nucleotides 680 to 910 as described previously (18). The HA gene, segment 4, specifying the HA1 domain of A/H3N2 virus, was amplified with segment-specific primers as described elsewhere (3).

**Sequencing analysis.** PCR products were then sequenced to examine mutations at amino acid positions 26, 27, 30, and 31 in the transmembrane region of M2 protein. The templates were labeled by cycle sequencing reactions with fluorescent dye terminators from the BigDye Terminator cycle sequencing kit, version 3.1, according to the instructions of the manufacturer (Applied Biosystems, Foster City, CA), and the products were sequenced using an ABI 3100 automatic sequencer (Applied Biosystems). Multiple-alignment data were analyzed using BioEdit (version 7.0.7) software. The HA sequences were assembled, aligned, and edited using BioEdit and MEGA 3.1 software (15). A phylogenic tree based on the HA genes was constructed by using the neighbor-joining method, and bootstrap analysis ($n$ = 1,000) was carried out to determine the best-fitting tree for each gene (29). Major branches with bootstrap values of >70% were assessed as distinct clades. Vaccine strain sequences, as well as sequences from viruses collected in Taiwan and Vietnam and on the main islands of Japan during the study period, were downloaded from BioHealthBase (http://www.biohealthbase.org/GSearch/statsAutomation.do?decorator=Influenza) and were also included in the analysis as references.

**Nucleotide sequence accession numbers.** The HA gene nucleotide sequences obtained in this study were submitted to the DNA Data Bank of Japan (DDBJ [http://www.ddbj.nig.ac.jp/Welcome-e.html/]) and assigned accession numbers AB378379 to AB378483.

## RESULTS

**Epidemiology of influenza in Okinawa.** The epidemic curve of influenza cases from the National Influenza Surveillance in Okinawa Prefecture showed regular winter peaks during the period from December to March and variable summer peaks during the period from May to August between January 2001 and March 2007 (Fig. 1). Summer peaks were observed in four (66.7%) of six seasons, namely, in 2001, 2002, 2005, and 2006.

As for virus surveillance, a total of 417 influenza viruses were collected at the Okinawa Prefectural Institute of Health and Environment from January 2001 to March 2007. Among these viruses, the numbers of influenza A/H1N1, A/H3N2, and B virus isolates were 40, 216, and 161, respectively. Winter circulation data showed the mixed circulation of A/H1N1, A/H3N2, and B viruses in 2001 and 2002, A/H1N1 and A/H3N2 viruses in 2003 and 2004, and A/H3N2 and B viruses in 2005 and the circulation of only A/H3N2 virus in 2006 and 2007 (Fig. 1). The circulating types and subtypes were similar to those observed on the main islands of Japan during the same periods (http://www.nih.go.jp/niid/index.html). Summer circulation data showed that, in 2001 and 2002, influenza A/H3N2 and B viruses cocirculated but the predominant strain was influenza B virus. In 2005, only A/H3N2 was circulating. In 2006, influenza A/H1N1 and B viruses cocirculated.

**Prevalence of amantadine-resistant strains.** A total of 73 (33.3%) of 216 A/H3N2 isolates were amantadine resistant, but none of the 40 A/H1N1 isolates were resistant (Table 1; Fig. 1). All the amantadine-resistant A/H3N2 isolates had an amino acid change from serine to asparagine at position 31 (Ser31Asn) in the M2 protein, as determined by genetic sequencing. After the first detection of an amantadine-resistant strain in Okinawa in January 2003, the prevalence of amantadine resistance increased from 7.3% in winter 2003 to 33.3% in winter 2005 and dramatically increased to 90.0% in summer 2005 (Table 1). The rates remained high (80 to 90%) thereafter until winter 2007.

**Phylogenetic tree analysis of A/H3N2 isolates.** A phylogenetic tree analysis was carried out for sequences of the HA1 domains of 73 A/H3N2 isolates obtained from January 2001 to May 2007 in Okinawa, including 8 vaccine strains and viruses collected from Taiwan, Vietnam, and the main islands of Japan during the study period (Fig. 2). These viruses formed two monophyletic lineages, A/Panama/2007/1999-like strains and A/Fujian/411/2002-like strains. The first A/Fujian/411/2002-like strain (A/Okinawa/45/2002) was isolated on 22 August 2002. The A/Fujian/411/2002-like strains could be subdivided into four period groups (I to IV) by influenza season: group I, isolated between November 2002 and March 2003; group II, isolated between November

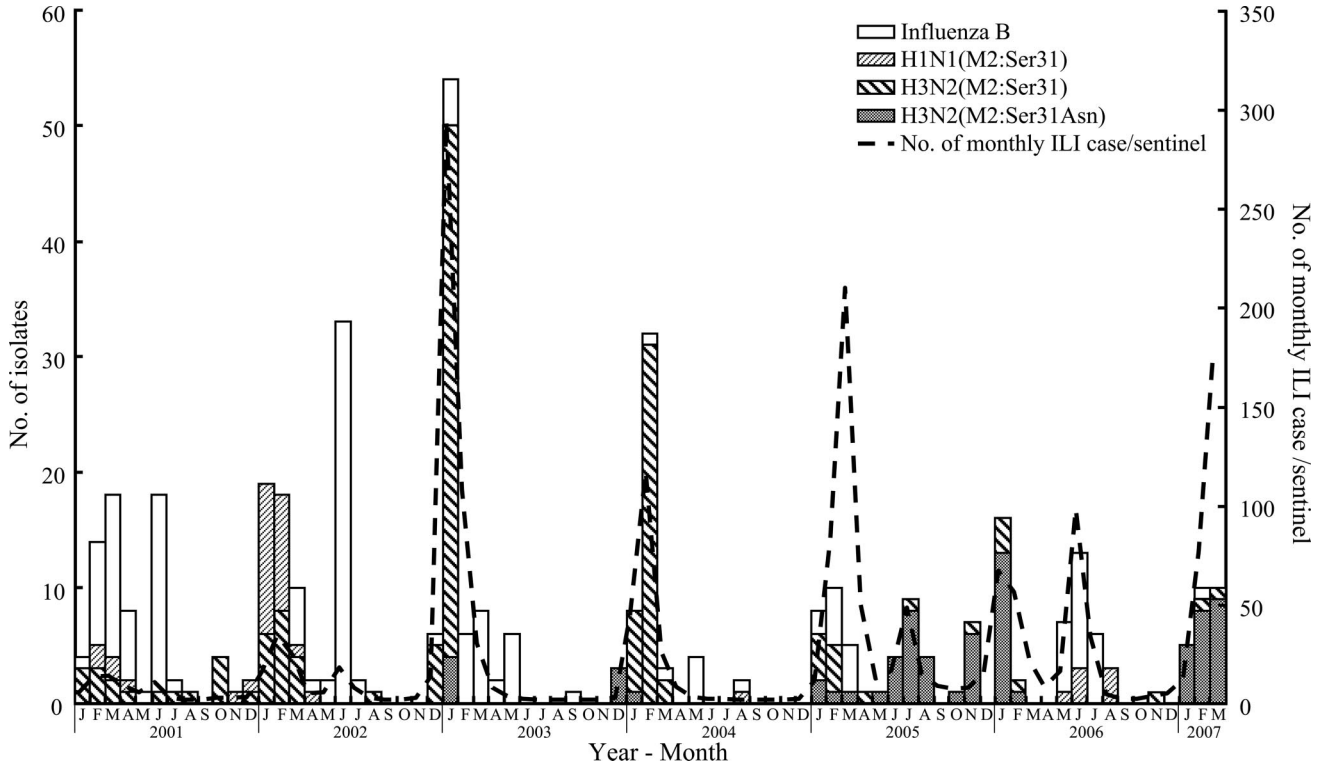


FIG. 1. Influenza epidemiological and laboratory data for the period from January 2001 to March 2007 in Okinawa, Japan. The numbers of influenza cases identified at designated clinical sentinels in Okinawa are plotted in a broken line. The monthly numbers of influenza virus isolates recovered at the microbiology division of the Okinawa Prefectural Institute of Health and Environment are also shown. Weekly data from the source were converted to monthly values. Names of months are abbreviated by first letters.

2003 and March 2004; group III, isolated between November 2004 and March 2005; and group IV, isolated between April 2005 and March 2007. In general, each group consisted of an independent cluster of amantadine-resistant viruses with a bootstrap value of >70%. Groups II and III included one resistant isolate each, A/Okinawa/8/2004 and A/Okinawa/11/2005, respectively. In group IV, all of the amantadine-resistant viruses from the 2005 summer season (A/Okinawa/14/2005 to A/Okinawa/32/2005) and the November 2005-to-March 2006 season (A/Okinawa/33/2005 to A/Okinawa/38/2005 and A/Okinawa/1/2006 to A/Okinawa/19/2006) were exclusively clustered in the so-called clade N (25, 26), with the dual substitutions Ser193Phe and Asp225Asn in the HA1 region of HA. In the November 2006-to-March 2007 season, clade N also comprised sensitive strains (classified as clade N-s).

TABLE 1. Prevalence of amantadine resistance among influenza A viruses by season in Okinawa, January 2001 to May 2007

| Year | Season | No. of resistant isolates[a]/total no. of isolates (%) of type: | |
|---|---|---|---|
| | | H3N2 | H1N1 |
| 2001 | Winter (Jan.–Mar. 2001) | 0/8 (0.0) | 0/4 (0.0) |
| | Summer (Apr.–Oct. 2001) | 0/8 (0.0) | 0/1 (0.0) |
| 2002 | Winter (Nov. 2001–Mar. 2002) | 0/19 (0.0) | 0/26 (0.0) |
| | Summer (Apr.–Oct. 2002) | 0/1 (0.0) | 0/1 (0.0) |
| 2003 | Winter (Nov. 2002–Mar. 2003) | 4/55 (7.3) | – |
| | Summer (Apr.–Oct. 2003) | – | – |
| 2004 | Winter (Nov. 2003–Mar. 2004) | 4/44 (9.1) | – |
| | Summer (Apr.–Oct. 2004) | – | 0/1[b] (0.0) |
| 2005 | Winter (Nov. 2004–Mar. 2005) | 4/12 (33.3) | – |
| | Summer (Apr.–Oct. 2005) | 18[c]/20 (90.0) | – |
| 2006 | Winter (Nov. 2005–Mar. 2006) | 21/24 (87.5) | – |
| | Summer (Apr.–Oct. 2006) | – | 0/7 (0.0) |
| 2007 | Winter (Nov. 2006–Mar. 2007) | 21/25[d] (84.0) | – |
| Total | | 72/216 (33.2) | 0/40 (0.0) |

[a] All amantadine-resistant viruses had the Ser31Asn mutation in the M2 transmembrane region. –, no isolates obtained.
[b] Isolate from a person who returned from the Philippines in August 2004.
[c] Including one isolate from a person who returned from Vietnam in June 2005.
[d] Including one isolate from a person who returned from Vietnam in November 2006.

**Amino acid differences in the HA1 subunits of A/H3N2 isolates collected from 2002 to 2007.** Amino acid sequences in the HA1 regions of A/H3N2 isolates obtained from 2002 to 2007 were compared with the corresponding sequence from the vaccine strain A/Fujian/411/2002, and the HA1 sequences of representative amantadine-sensitive and -resistant viruses in each period group were also compared (Table 2). In group I, resistant viruses had an amino acid substitution at position 291 in comparison with the sequence of the A/Fujian/411/2002 strain, while sensitive viruses had no amino acid substitution at this position. In group II, sensitive and resistant viruses had three amino acid substitutions, at positions 159, 189, and 227, and resistant viruses had additional substitutions at positions 140, 198, and 214. In group III, sensitive viruses had two amino acid substitutions, at positions 145 and 226, in addition to those in the group II viruses, and resistant viruses had the same substitutions except at position 227, where the amino acid reverted to that


FIG. 2. Phylogenetic analysis of the HA1 domains of the HA genes (sequences of 841 nucleotides) of influenza A/H3N2 virus isolates collected in Okinawa, Japan, from January 2001 to March 2007. ■ indicates amantadine-resistant (Am-R) viruses; amantadine-sensitive (Am-S) viruses are unmarked. Okinawa isolates are shown in bold, and reference vaccine strains are shown in bold italics. Sequences of viruses from the regions and main islands of Japan were obtained from the BioHealthBase. Bootstrap values of >70% are shown.

in the group I viruses. In group IV, sensitive viruses had two amino acid substitutions, at positions 112 and 173. Resistant strains within clade N that were first isolated in the 2005 summer season had two amino acid substitutions, at positions 193 and 225. Among the November 2006-to-March 2007 season isolates in group IV, resistant strains had two additional amino acid substitutions, at residues 50 and 140, and sensitive strains clustering within clade N (clade N-s)

TABLE 2. Comparison of amino acid substitutions in the HA1 regions of A/H3N2 isolates collected from December 2002 to March 2007

| Period group | Strain[a] | Amino acid[b] at position: | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 45 | 50 | 112 | 124 | 140 | 142 | 145 | 157 | 159 | 173 | 189 | 193 | 198 | 214 | 225 | 226 | 227 | 291 |
| Reference | **Fujian/411/2002** | S | G | V | S | K | R | K | L | Y | K | S | S | A | I | D | V | S | G |
| I | Okinawa/15/2003 | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| | Okinawa/26/2003 ■ | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | – | N |
| II | Okinawa/2/2004 | – | – | – | – | – | – | – | – | F | – | N | – | – | – | – | – | P | – |
| | Okinawa/52/2003 ■ | – | – | – | – | Q | – | – | – | F | – | N | – | T | V | – | – | P | – |
| III | Okinawa/8/2005 | – | – | – | – | – | – | N | – | F | – | N | – | – | – | – | I | P | – |
| | Okinawa/6/2005 ■ | – | – | – | – | – | – | N | – | F | – | N | – | – | – | – | I | – | – |
| IV[c] | Okinawa/22/2005 | – | – | I | – | – | – | N | – | F | E | N | – | – | – | – | I | P | – |
| | Okinawa/35/2005 ■ | – | – | – | – | – | – | N | – | F | – | N | F | – | – | N | I | P | – |
| | Okinawa/12/2007 ■ | – | E | – | – | I | – | N | – | F | – | N | F | – | – | N | I | P | – |
| | Okinawa/7/2007[d] | N | – | – | – | – | G | N | S | F | E | N | F | – | – | N | I | P | – |

[a] Bold type indicates the reference vaccine strain. Black squares denote amantadine-resistant strains.
[b] Amino acid substitutions with respect to the sequence of the reference strain Fujian/411/2002. –, no difference from the sequence of the reference strain.
[c] Period group IV includes the clade N strains.
[d] Amantadine-sensitive strain which belonged to clade N-s in the clade N lineage.

had four additional amino acid substitutions, at positions 45, 142, 157, and 173.

## DISCUSSION

From January 2001 to May 2007, we conducted an epidemiological study of influenza in Okinawa Prefecture, located in the subtropical zone of Japan. Our study indicated that the seasonality of influenza in Okinawa showed two peaks (from December to March and May to August). This pattern is characteristic of influenza circulation in subtropical areas, as we reported previously for North Vietnam, which has two peaks: in the hot rainy season and in winter (16). However, in this study, Okinawa sometimes did not show clear double peaks. Taiwan is the nearest neighboring country to Okinawa and has the same climate. Influenza viruses were isolated almost throughout the year in Taiwan, with hot rainy season and winter peaks (12, 13, 17, 31). Between late 2001 and early 2007, Taiwan had three summer peaks, with a predominance of influenza B virus in 2002 and A/H3N2 virus in 2004 and 2005 (12, 13, 17, 31). In the years in which Okinawa had summer peaks, the circulating types and subtypes were similar to those circulating during the same season in Taiwan. On the other hand, the epidemic types circulating in winter in Okinawa and Taiwan coincided in only four (57.1%) of seven seasons (12, 13, 17, 31), while those in Okinawa and on the main islands of Japan coincided in six (85.7%) of seven winter outbreaks. Thus, the epidemic types in Okinawa and on the main islands of Japan coincided mainly in the winter season, and those in Okinawa and Taiwan coincided mainly in the summer season.

An A/Fujian/411/2002-like virus was isolated in summer in Okinawa several months prior to the predominant circulation of this strain in winter on the main islands of Japan and in Okinawa. Clade N amantadine-resistant viruses circulating in 2005 also emerged in Okinawa several months before their detection on the Japanese main islands. The early detection of predominant strains in Okinawa compared to the detection of such strains on the Japanese main islands was also seen for influenza B virus (in 2001, 2002, and 2006) and A/H1N1 virus (in 2006) according to National Influenza Surveillance data (http://idsc.nih.go.jp/iasr/index-j.html). Therefore, epidemic strains circulating in Japan in winter may appear in Okinawa almost half a year ahead of their detection on the main islands.

During the period between 1991 and 2000, Okinawa had only one summer peak, in 1995 (unpublished data collected at the Okinawa Prefectural Institute of Health and Environment), but the summer peaks became more frequent after 2001. The low numbers of reports of influenza during the summer in Okinawa throughout the 1990s were due probably to the low level of attention from clinicians toward influenza or to the incompletely subtropical climate at the border of subtropical and temperate zones. Global warming has been progressing over the last century (32); a temperature rise of ~1.0°C in Japan has been observed consistently during the past 100 years. Both rotavirus and influenza virus infections occur in the winter season in temperate areas. The peak of rotavirus infections in Japan has shifted gradually from winter to early spring for unknown reasons (34) The increased tendency toward summer peaks in Okinawa in recent years may also be associated with climatic changes. Furthermore, the unique geographical position of Okinawa allows for the study of the change in the seasonal pattern of influenza from temperate to subtropical climates.

The emergence of resistance following treatment with amantadine occurred only transiently and the levels of circulating resistant viruses were less than 4% in Japan, Europe, and the Americas until 2004 (4, 5, 14, 33, 35, 40). In Okinawa, no amantadine-resistant strains were found in 2001 and 2002, but the prevalence increased to 7.3% at the beginning of 2003 and surged to 90% in 2005. The high levels of amantadine resistance in influenza A/H3N2 virus strains in the community were not related to domestic amantadine use, since the total yearly consumption of the drug was steady or decreased, as in other prefectures in Japan (27). In other parts of the world, unusu-

ally high prevalences of amantadine resistance have been most prominent in China, Hong Kong, and Taiwan since 2003, after the outbreak of severe acute respiratory syndrome and outbreaks of avian influenza, probably due to the excessive use of amantadine (4). Thus, Okinawa's early rise of resistance compared to that on the Japanese main islands indicated a strong influence from China or Taiwan on Okinawa in terms of influenza transmission. A recent study of avian influenza A/H5, A/H6, A/H7, and A/H9 virus subtypes also showed an increased level of resistance during the period from 2000 to 2004 (11). The increased resistance of some avian influenza viruses to amantadine was suggested previously to be derived from the excessive use of the drug by farmers in China (8). We assume that although the mechanism of generation of amantadine resistance in community-circulating strains has not been fully elucidated, this high frequency of resistance in human strains was not caused solely by an excess use of the drug for humans in each country but that food or environmental contamination also played a role. We would like to emphasize that the uncontrolled use of amantadine in humans and birds poses a risk of sustained circulation of amantadine-resistant strains and, thus, monitoring the drug consumption is important.

The phylogenic analysis of the HA1 genes of A/H3N2 strains from Okinawa showed four groups corresponding to periods between 2002 and 2007, and each group consisted of resistant and sensitive clusters. We previously demonstrated that the high incidence of resistant virus is related to the appearance of clade N (26–28), which possesses double mutations in HA1, at positions 193 and 225. These positions are near receptor binding sites (37) and are possibly associated with a high degree of transmission fitness of influenza viruses in the clade (19, 20). In this study, we demonstrated the tight grouping of the HA1 domains of the HA genes in association with the possession of resistance mutations in the M2 genes of A/H3N2 viruses, even those obtained before the appearance of clade N. However, most of these mutations (those at HA amino acid positions 198, 214, 227, and 291) in the HA1 domains of groups I to III were not located at antigenic and/or receptor binding sites, unlike those in clade N of group IV (37). We need to elucidate the genetic association among HA and M2 genes and/or other genes in the generation of community-circulating amantadine-resistant strains and the relationship of these genes to the fitness of the resistant strains.

The clade N amantadine-resistant viruses have started to circulate since May 2005 in Okinawa, as in various East and Southeast Asian countries (2). Resistant viruses caused a small local influenza outbreak in Nagasaki in September 2005 (25), and then the circulation of these viruses in various parts of Japan from November 2005 to April 2006 was observed (27, 39). In our previous report, the kriging map from a geographical information system showed nationwide influenza epidemic patterns spreading in concentric circles from west-central Japan to northeastern Japan (30). Another report indicated that new variant influenza A/H3N2 virus strains were first detected in East and Southeast Asia and China and were then seeded into the temperate regions, including Japan (24). These results support our views that influenza infections spread to Japan from its western and southern neighbors every year. Thus, the surveillance of influenza virus circulation in Okinawa is potentially important to monitor the emergence of new influenza virus variants and to elucidate the dynamics of the seasonal pattern of influenza in a border area between temperate and subtropical regions.

Amantadine-resistant A/H1N1 virus was not detected in Okinawa during our study period until March 2007, whereas it was detected on a main island of Japan in February 2007 (28). The lack of resistance in summer 2006 in Okinawa may be ascribed to low levels of circulation of amantadine-resistant A/H1N1 strains in Southeast Asia during the same period (1). However, the question of why A/H1N1 viruses were not isolated in Okinawa from September 2006 to March 2007 remains. This result may reflect the finding that the types and subtypes of influenza virus strains circulating in Okinawa and on the main islands of Japan in winter time do not always coincide. So far, we have not detected oseltamivir-resistant A/H1N1 strains in Okinawa, but further analysis of the most recently isolated strains is warranted to clarify this important aspect.

In conclusion, as epidemic strains and community-circulating amantadine-resistant influenza viruses were detected earlier in Okinawa than on the main islands of Japan through surveillance activities, we can emphasize that Okinawa is a very important sentinel site to monitor influenza infections in Japan, especially those with strains circulating in Asia in summer. Furthermore, the World Health Organization reported a high incidence of oseltamivir-resistant A/H1N1 strains in many countries (38), as well as amantadine-resistant influenza A virus (2, 5, 6, 16, 26-28, 39). We assume that the molecular epidemiology data on influenza in both human and avian species collected over several seasons in multiple countries provide important information to elucidate the spatiotemporal movements of viruses in the near future.

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